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A Point Mutation in the Human Cytomegalovirus DNA Polymerase Gene Selected *in Vitro* by Cidofovir Confers a Slow Replication Phenotype in Cell Culture

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In cell culture, cidofovir (CDV) was used to select a human cytomegalovirus (HCMV) strain with decreased drug susceptibility. The genotypic characterization of this virus revealed a single base substitution resulting in a K513N amino acid alteration in the viral DNA polymerase (UL54). Performed in parallel, the selection of HCMV for replication in the presence of ganciclovir (GCV) selected an M460V substitution in the phosphotransferase (UL97), as well as a K513N/V812L double substitution in DNA polymerase. Neither of the two DNA polymerase mutations has been previously identified in HCMV drug-resistant strains. To precisely elucidate their role in drug resistance, corresponding recombinant mutant viruses were generated by recombination of nine overlapping viral DNA fragments. The K513N recombinant virus showed 13- and 6.5-fold decreased susceptibility to CDV and GCV *in vitro*, respectively, compared with the wild-type recombinant virus. Mutation V812L was associated with a moderate (2–3-fold) decrease in susceptibility to CDV, GCV, foscarnet, and adefovir. A multiplicative interaction of the K513N and V812L mutations with regard to the profile and level of drug resistance was demonstrated in recombinant virus expressing both mutations. *In vitro* replication kinetic experiments revealed that the K513N mutation significantly decreased HCMV replication capacity. Consistent with this finding, the K513N mutant DNA polymerase exhibited reduced specific activity in comparison with the wild-type enzyme and was severely impaired in its 3′-5′ exonuclease function. Unexpectedly, the K513N mutant enzyme showed no decrease in susceptibility to CDV-diphosphate or GCV-triphosphate. However, the K513N mutation decreased the susceptibility to CDV and GCV of the *ori*Lyt plasmid replication in the transient transfection/infection assay, suggesting that the DNA replication of the K513N mutant virus is less sensitive to the corresponding inhibitors.

INTRODUCTION

Since the beginning of the acquired immune deficiency syndrome (AIDS) epidemic, human cytomegalovirus (HCMV) has been a major infectious pathogen, causing retinitis and other opportunistic diseases in a significant number of the patients with late-stage AIDS (Jacobson and Mills, 1988). Although recent progress in the treatment of human immunodeficiency virus infections has resulted in a marked decline in the incidence of opportunistic diseases, HCMV infections remain a concern in patients with AIDS. In addition, after bone marrow or solid organ transplantation, immunosuppressed patients may have HCMV infections (Rubin, 1990), requiring either anti-HCMV prophylaxis or treatment. However, due to the chronic character of anti-HCMV therapy, resistance may develop, which has been associated with clinical failure and disease progression (Erice *et al.*, 1989; Wolf *et al.*, 1995).

Resistance to ganciclovir (GCV), one of the three currently approved anti-HCMV drugs (Crumpacker, 1996), has been shown to be associated with specific sequence alterations in the viral UL97 gene (Sullivan *et al.*, 1992; Biron and Baldanti, 1996; Baldanti *et al.,* 1998). The product of this gene has been proposed to catalyze the first step in the metabolic activation of GCV (Littler *et al.*, 1992). In addition, specific mutations in the HCMV DNA polymerase gene (UL54) confer decreased susceptibility to GCV. The presence of UL54 alterations only in GCV-resistant strains is rare; most often, they are present in combination with at least one UL97 mutation (Biron and Baldanti, 1996; Smith *et al.*, 1997; Sullivan *et al.,* 1993). Viruses with both UL54 and UL97 mutations are selected primarily during extended GCV therapy and exhibit high-level GCV resistance (Smith *et al.,* 1997).

Cidofovir (CDV) and foscarnet (PFA) are two additional clinically available drugs for the treatment of HCMV infections (Crisp and Clissold, 1991; Lalezari, 1997). When PFA was administered as a first-line therapy, selection of resistant viruses was not detected (SOCA, 1997). However, PFA-resistant strains carrying specific genotypic alterations in the UL54 gene can arise when PFA treatment follows GCV therapy (Baldanti *et al.,* 1995a, 1996; Chou *et al.,* 1997). Generation of CDV-resistant strains during CDV therapy has not been reported to date (Cherrington *et al.,* 1996, 1998). However, strains with decreased susceptibility to GCV due to UL54 mutations exhibit cross-resistance to CDV (Lurain *et al.*, 1992; Smith *et al.*, 1997; Sullivan *et al.,* 1993). On the other hand,

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because CDV is activated through phosphorylation catalyzed by cellular enzymes (Cihlar and Chen, 1996), UL97 mutants resistant to GCV still remain susceptible to CDV (Smith *et al.,* 1997).

All three currently available anti-HCMV drugs target viral DNA polymerase. Both CDV-diphosphate (CDVpp) and GCV-triphosphate (GCVppp), the active metabolites of the respective drugs, are effective competitive polymerase inhibitors (Hitchcock *et al.,* 1996; Mar *et al.,* 1985a). PFA, on the other hand, functions directly as a noncompetitive inhibitor (Öberg, 1989).

Additional antiviral drugs targeting HCMV DNA polymerase are being evaluated in clinical trials. Among them, adefovir [ADV; 9-(2-phosphonomethoxyethyl)adenine] and lobucavir (LBV; cyclobut-G) efficiently suppress HCMV replication *in vitro,* and their active forms, ADV-diphosphate and LBV-triphosphate, have been shown to be potent inhibitors of HCMV DNA polymerase (Tenney *et al.,* 1997; Xiong *et al.,* 1997a). It has been demonstrated by generation and characterization of recombinant viruses that specific UL54 mutations can decrease virus susceptibility to either of these two agents (Cihlar *et al.,* 1998).

In vitro selection of viruses with decreased drug susceptibility is a useful tool for mapping drug resistance-associated genotypic alterations, evaluating cross-resistance profiles, and elucidating molecular mechanisms of antiviral activity. HCMV strains selected in cell culture in the presence of GCV were shown to harbor both UL97 and UL54 mutations (Lurain *et al.,* 1992; Sullivan *et al.,* 1993). In the UL54 gene, point mutations leading to F412V, L501I, or A987G amino acid substitutions in DNA polymerase were identified in independently selected viruses. These mutations also resulted in cross-resistance to CDV (Lurain *et al.,* 1992; Sullivan *et al.,* 1993). However, no detailed genotypic and phenotypic characterization of a resistant HCMV strain selected by CDV has been presented to date.

In this study, we describe an *in vitro* selection of drug-resistant HCMV in the presence of CDV. For comparison, a simultaneous selection in the presence of GCV was performed. Genotypic alterations in the selected viral strains were identified and their association with drug resistance was confirmed by generation of corresponding mutant recombinant viruses. Characterization of these recombinants revealed that the mutation selected for by CDV diminished HCMV replication capacity in cell culture. In addition, further data concerning the molecular mechanisms of CDV resistance are presented.

RESULTS

Mutation K513N in the UL54 gene is selected *in vitro* by both CDV and GCV

To characterize the molecular aspects of CDV resistance, an HCMV strain with decreased susceptibility to CDV was selected in cell culture. For comparison, a parallel selection in the presence of GCV was also performed. In the initial step, laboratory strain AD169 was cultured in the presence of 0.5 μM CDV or 3 μM GCV (i.e., at drug concentrations corresponding approximately to their respective IC_{50} values). Over a period of 10 months, the viruses were passaged every 7–14 days, when significant cytopathic effect was observed. The drug concentrations were gradually increased every second to fourth passage depending on the level of virus replication. After ~20 passages in the presence of CDV, a virus was obtained that was able to replicate at 16 μM CDV, which is 32-fold higher than the CDV IC_{50} value of the parental virus. An equal number of passages in the presence of GCV selected for a virus replicating at 300 μM GCV (i.e., at concentration exceeding 60-fold the GCV IC_{50} value for the parental AD169 strain). From each of these independently selected virus pools, two strains were isolated by four rounds of plaque purification and assayed for their susceptibility to CDV and GCV. Table 1 shows that the strains selected for by CDV (CDV.R1 and CDV.R2) exhibited a 16–24-fold and ~10-fold reduced susceptibility to CDV and GCV, respectively. Comparatively, the GCV-selected strains (GCV.R1 and GCV.R2) demonstrated more significant decrease in their susceptibility to both CDV (20–32-fold) and GCV (55–60-fold). Genotypic analysis revealed an identical G-to-T mutation at nucleotide 1539 in the UL54 gene from both CDV- and GCV-selected strains compared with the AD169 sequence. This alteration results in a Lys-to-Asn substitution at codon 513 located in the polymerase conserved δ-region C (Fig. 1). In addition, both the GCV-selected strains also carried a G-to-T mutation at nucleotide 2434 corresponding to a Val-to-Leu amino acid alteration at codon 812 residing within the UL54 conserved region III.

Sequencing of the UL97 phosphotransferase gene revealed an ATG (Met)-to-GTG (Val) substitution at codon

TABLE 1

Drug Susceptibilities of HCMV Strains Selected *in Vitro* by CDV or GCV

	IC_{50} (μM)[a]	
Virus	CDV	GCV
AD169	0.5	5.0
CDV selected		
CDV.R1	12 (24)[b]	50 (10)
CDV.R2	8 (16)	55 (11)
GCV selected		
GCV.R1	10 (20)	275 (55)
GCV.R2	16 (32)	300 (60)

[a] The IC_{50} values represent the mean from at least four independent determinations. In all cases, the standard error was <35%.

[b] The numbers in parentheses indicate the fold increase above the corresponding IC_{50} value of the parental AD169 strain.

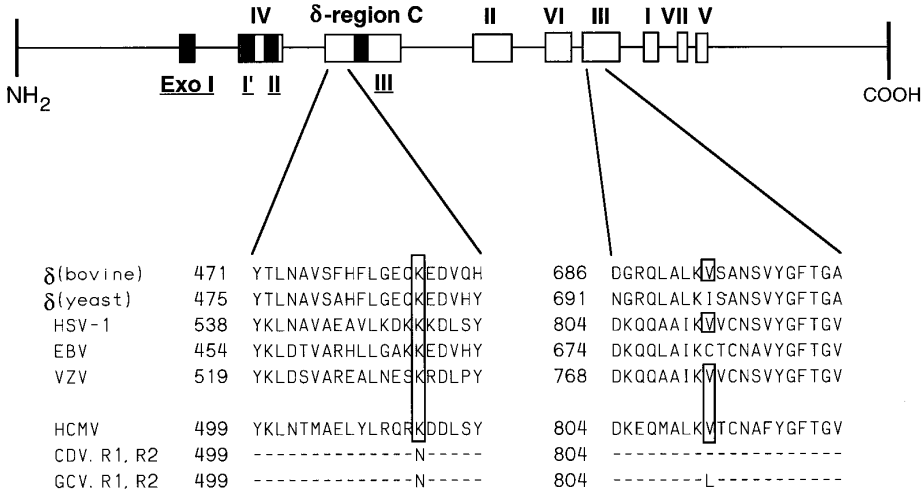

FIG. 1. Amino acid substitutions in HCMV DNA polymerase selected *in vitro* by CDV or GCV. In the linear map of polymerase polypeptide, conserved regions (I-VII and δ-region C) and exonuclease domains (Exo I-III), respectively, are shown. Their boundaries with respect to amino acid numbering of HCMV DNA polymerase have been defined as follows (Bernad *et al.,* 1988; Blanco *et al.,* 1991; Hwang *et al.,* 1992; Simon *et al.,* 1991; Wong *et al.,* 1988; Zhang *et al.*, 1988): IV (379–421), δ-region C (492–587), II (696–742), VI (771–790), III (805–845), I (905–919), VII (962–970), V (978–988), ExoI (295–312), ExoI′ (376–393), ExoII (404–418), and ExoIII (533–545). The alignment of amino acid sequences with boxes showing homology at mutated codons is based on previously published data (Ito and Braithwaite, 1991; Wong *et al.,* 1988; Zhang *et al.,* 1991).

460 in both the GCV.R1 and GCV.R2 viruses. As expected, no mutation was detected in the UL97 gene of the CDV.R1 or CDV.R2 virus.

Mutation K513N is associated with both CDV and GCV resistance

Although the K513E and K513R substitutions in the UL54 gene were identified previously in HCMV strains with decreased drug susceptibility (Smith *et al.,* 1997), the K513N is a novel alteration that has not been identified before in any drug-resistant isolate. Likewise, the V812L mutation has not been previously described. To precisely elucidate the association of these two UL54 substitutions with drug resistance, recombinant viruses expressing independently each of these alterations were constructed, and their drug susceptibility profiles were determined. The data in Table 2 show that an introduction of the K513N mutation into the UL54 gene resulted in a ~13- and 6-fold decrease in the virus susceptibility to CDV and GCV, respectively, compared with the recombinant virus with WT UL54 gene. When tested against ADV, the K513N recombinant virus exhibited a 3-fold increase in susceptibility to ADV compared with the wild-type (WT) virus. On the other hand, the V812L substitution conferred moderate, 2- to 3-fold decrease in susceptibility to CDV, GCV, PFA, and ADV. Interestingly, both the K513N and V812L mutant viruses remained WT with respect to their susceptibility to LBV.

To accurately examine the mutual interaction of the K513N and V812L mutations as well as the contribution of the K513N/V812L double mutation to the final drug resistance of GCV.R1 and GCV.R2, a recombinant virus expressing both of these alterations was generated. The drug susceptibility assays revealed 10-fold reduced susceptibility to GCV and a >30-fold decrease in sensitivity to CDV compared with the WT

TABLE 2

Drug Susceptibilities of Recombinant HCMVs Expressing UL54 Mutations Selected by CDV and GCV

	IC_{50} (μM)[a]				
UL54 substitution	CDV	GCV	PFA	ADV	LBV
None	0.75	3.5	45	42	3.8
K513N	9.4 (12.5)[b]	21 (6.0)	50 (1.1)	14 (−3.0)	3.5 (0.9)
V812L	2.4 (3.2)	8.8 (2.5)	130 (2.9)	122 (2.9)	5.7 (1.5)
K513N/V812L	25.5 (34)	34 (9.8)	135 (3.0)	26 (−1.6)	3.8 (1.0)

[a] Average from at least three determinations with two independently generated recombinant viruses. In all cases, the standard error was <35%.
[b] Underlined numbers indicate a significant (i.e., at least 2-fold) change in HCMV drug susceptibility. Positive and negative numbers in parentheses represent fold increase and decrease, respectively, in IC_{50} value relative to the recombinant virus with wild-type UL54 sequence.

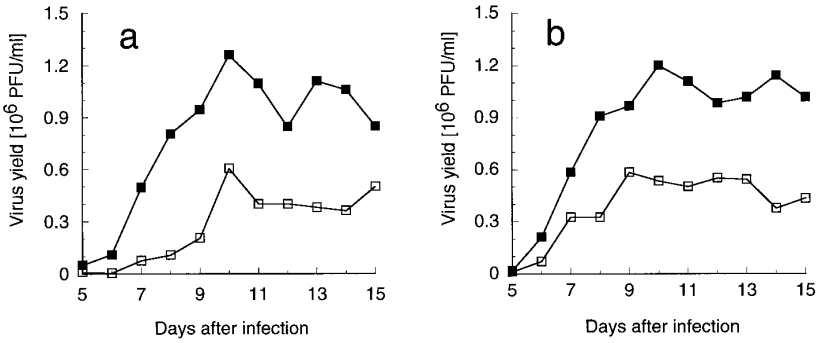

FIG. 2. Influence of the K513N polymerase substitution on HCMV replication capacity in cell culture. Confluent NHDFs in 12-well plates were infected either with the WT (■) or K513N (□) recombinant HCMV at 50–60 pfu/well, and the yield of extracellular virus was compared by titration of culture media. (a) and (b) show the representative growth kinetics of independently generated recombinants.

recombinant (Table 2). In addition, this recombinant mutant virus exhibited a 3-fold increase in PFA IC_{50} value, which represents a shift in PFA susceptibility similar to that associated with the V812L mutation alone. Overall, these data suggest a multiplicative interaction of K513N and V812L mutations with respect to the final drug susceptibility of the K513N/V812L double mutant virus.

The K513N mutant virus is replication impaired in cell culture

Because DNA polymerase is an essential enzyme for HCMV replication, specific mutations in the UL54 gene could influence the virus replication capacity. Compared with the WT virus, certain PFA-resistant HCMV mutants harboring UL54 mutations were found to replicate slower in cell culture (Baldanti *et al.,* 1996). Similarly, herpes simplex virus type 1 (HSV-1) strains carrying specific mutations in the DNA polymerase gene also exhibited a slow-growth phenotype *in vitro* (Hwang *et al.,* 1997). Based on these findings, the influence of the CDV-selected K513N polymerase alteration on the rate of HCMV replication in cell culture was examined.

In the replication kinetic experiments, production of extracellular virus was determined after infection of normal human dermal fibroblasts (NHDFs) with either the WT or K513N mutant recombinant. Rather than performing a single-cycle replication experiment after a high m.o.i., the cells were infected at a lower m.o.i., and virus production was determined during several rounds of viral replication. Figure 2 shows a comparison of the yield of both viruses during a period of 15 days after infection. The first increase in the level of extracellular virus was detectable at day 5. Subsequently, production of both viruses gradually increased, reaching a plateau by day 9. However, during the entire experiment, two independently generated K513N mutant recombinants showed a markedly lower level of virus production compared with the two WT recombinants, indicating that the K513N mutation decreased *in vitro* HCMV replication capacity.

K513N DNA polymerase exhibits reduced specific activity and lacks the 3′-5′ exonuclease activity

To further study the molecular mechanism of the K513N mutant slow replication phenotype, functional characterization of the K513N DNA polymerase was undertaken. The WT and K513N enzymes were isolated from cells infected with the corresponding recombinant virus using a standard, reproducible procedure that has been previously shown to yield highly purified protein (Mar *et al*., 1985b). In addition, both enzymes were expressed using a coupled *in vitro* transcription/translation system (Cihlar *et al.,* 1997). The *in vitro* expression in reticulocyte lysate was successfully used by others for characterization of mutant forms of HCMV DNA polymerase (Ye and Huang, 1993) and HSV-1 DNA polymerase (Dorsky and Crumpacker, 1988). Figure 3 shows the immunoblot analysis of the WT and K513N mutant enzyme isolated from virus infected cells as well as the sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) analysis of the *in vitro* expression products. Both in the native enzyme preparations and in the *in vitro* expression reactions, DNA polymerase protein with a molecular mass of 140 kDa was detected with no apparent differences between the WT and mutant proteins.

To compare the catalytic capacity of the WT and K513N enzyme, a relative amount of both proteins was determined. Enzymes isolated from infected cells and *in vitro* expressed enzymes were quantified by using the immunoblot assay and direct autoradiography of ^{35}S-labeled proteins, respectively. Both assays exhibited a linear response in the range of protein quantification (Fig. 4). Determination of the DNA-dependent DNA polymerase activity with an identical amount of the WT and K513N enzyme revealed that both the isolated and *in vitro* expressed K513N polymerase showed lower relative specific activity compared with the corresponding WT enzymes (Table 3). The difference between the WT and K513N polymerase was more pronounced in the case of the *in vitro* expressed enzymes; this may be due to the fact that the purification procedure yields a complex

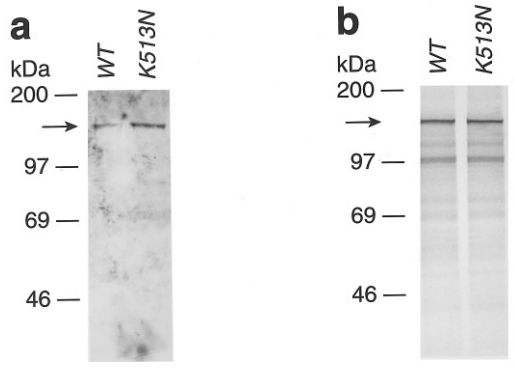

FIG. 3. Analysis of native isolated and *in vitro* expressed HCMV DNA polymerases. (a) After separation of enzyme aliquots by SDS–PAGE, immunoblot analysis with chemiluminescent detection of isolated enzymes was performed. The arrow indicates the 140-kDa polymerase protein. (b) Direct analysis of [^{35}S]methionine-labeled *in vitro* expressed enzymes after SDS–PAGE separation.

consisting of the UL54 catalytic subunit and the UL44 accessory protein (Mar *et al*., 1985b), whereas only the UL54 catalytic subunit was expressed in the reticulocyte lysate. It has been shown that the accessory protein may modify polymerase activity under certain conditions (Ertl and Powell, 1992; Weiland *et al*., 1994).

The polymerase region between amino acids 532 and 545 was identified as an ExoIII domain and based on homology with other replicative DNA polymerases was suggested to participate in the 3′-5′ exonuclease function of the enzyme (Bernad *et al.,* 1989; Blanco *et al.,* 1991). Because codon 513 is located in close vicinity to this region and it codes for an amino acid conserved among several other DNA polymerases (Fig. 1) (Zhang *et al.,* 1991), the 3′-5′ exonuclease activity of the WT and K513N mutant polymerase also was determined. The WT polymerase, both isolated from virus-infected cells and *in vitro* expressed, was capable of releasing [^{3}H]dTMP from the 3′-end of [^{3}H]oligo(dT) attached to linear double-strand DNA. However, the *in vitro* expressed K513N mutant enzyme exhibited exonuclease activity at ~2% of the level of the expressed WT enzyme, and the native K513N enzyme isolated from infected cells showed undetectable exonuclease activity (Table 3). These data indicate that the K513N substitution severely compromises the exonuclease function of HCMV DNA polymerase as well as affects its DNA-dependent DNA polymerase activity.

TABLE 3

Functional Comparison of the WT and K513N Mutant HCMV DNA Polymerase

Enzyme	Activity (units)[a]	
	DNA Polymerase	3′–5′ Exonuclease
Isolated from infected cells[b]		
WT	4.9 ± 0.9 (100%)	6.5 ± 1.1 (100%)
K513N	3.0 ± 0.5 (61%)	0 (0%)
In vitro expressed[c]		
WT	6.5 ± 1.1 (100%)	5.3 ± 1.3 (100%)
K513N	1.5 ± 0.5 (23%)	0.11 ± 0.06 (2%)

[a] One unit of the DNA polymerase and 1 unit of the 3′–5′ exonuclease activity corresponds with the incorporation of 1 pmol of dGTP into activated calf thymus DNA and with release of 1 pmol of dTMP from the single-stranded DNA substrate, respectively, per 1 h under the conditions specified in Materials and Methods. The data represent the mean with standard error values from two independent experiments.

[b] Enzymes isolated from infected cells were quantified by using the immunoblot assay as described in Materials and Methods. An equal amount of the WT and K513N mutant enzyme was used for their functional comparison.

[c] Quantification of the *in vitro* expressed enzymes was performed by direct autoradiography of the ^{35}S-labeled proteins as described in Materials and Methods. An equal amount of the WT and K513N mutant enzyme was used for their functional comparison. The background DNA polymerase and 3′–5′ exonuclease activities detected in the reticulocyte lysate accounted for <5% of those of the WT enzyme. The numbers represent values after subtraction of the corresponding background activities.

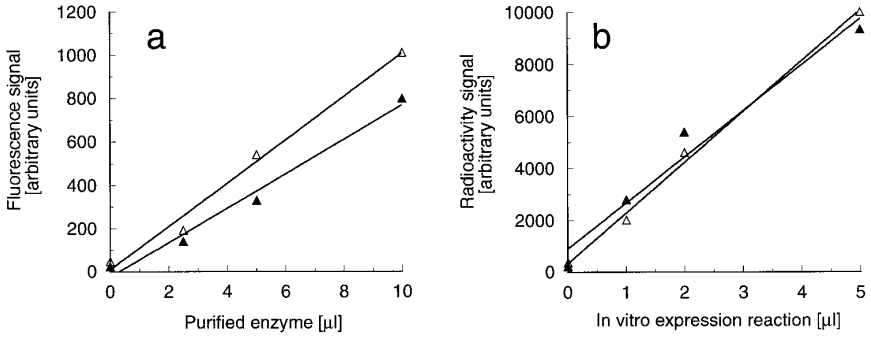

FIG. 4. Linear response of the assays used for relative quantitation of the WT (△) and K513N mutant (▲) HCMV DNA polymerase. (a) Varying amounts of enzymes isolated from virus-infected cells were separated on SDS–PAGE and electroblotted onto a polyvinylidene difluoride membrane. Subsequent immunoblot analysis and quantification was performed as described in Materials and Methods. (b) *In vitro* expression of both enzymes was performed in the presence of [^{35}S]methionine, and varying amounts of the expression reactions were separated on SDS–PAGE. The fixed and dried gel was scanned using the PhosphorImager system, and the bands corresponding to the full-length UL54 proteins were quantified.

TABLE 4

Sensitivity of the WT and Mutant HCMV DNA Polymerases Towards Anti-HCMV Inhibitors

	K_m (μM)[a]			K_i (μM)[a]			
Enzyme	dCTP	dGTP	dATP	CDVpp	GCVppp	PFA	ADVpp
WT	0.26	0.24	0.66	1.2 (4.6)[b]	0.085 (0.35)	3.5 (14.6)	0.074 (0.11)
K513N	0.31	0.26	0.38	1.5 (4.8)	0.11 (0.42)	3.6 (13.8)	0.044 (0.12)
V715M	0.78	0.39	1.14	2.9 (3.7)	0.25 (0.62)	45 (123)	0.48 (0.42)

[a] The data are averages from at least two independent experiments.
[b] The numbers in parentheses represent K_i/K_m values.

The K513N mutation decreases susceptibility of viral DNA replication to CDV and GCV

Using the enzymes isolated from cells infected with corresponding recombinant viruses, we investigated a potential correlation between the decreased drug susceptibility of the K513N mutant virus and the sensitivity of the K513N mutant polymerase toward corresponding inhibitors. Steady-state kinetic experiments were performed to determine the K_m values for dNTPs and the K_i values for CDVpp, GCVppp, PFA, and ADVpp. Enzyme sensitivities toward the inhibitors were compared based on the corresponding ratios of K_i to K_m.

Unexpectedly, no significant differences between the WT and K513N enzyme, particularly with regard to their sensitivities to CDVpp and GCVppp were found (Table 4). Similarly, no differences in drug susceptibility were found between the enzymes isolated from cells infected with the parental strain AD169 and the strain CDV.R1 (data not shown). To clarify whether this lack of correlation between virus and enzyme drug susceptibility was a more general HCMV phenomenon, drug susceptibility of the V715M mutant polymerase was characterized. It was shown previously that the V715M substitution conferred 10- and 5-fold decreased susceptibility to PFA and ADV, respectively (Baldanti *et al.,* 1996; Cihlar *et al.,* 1998). The enzyme was isolated from cells infected with the corresponding recombinant V715M mutant virus. In the enzyme preparation, a 140-kDa polymerase protein identical to that in the WT and K513N enzyme preparation was detected by immunoblot assay (data not shown). Interestingly, data in Table 4 show that the V715M mutant enzyme was ~9- and ~4-fold less susceptible to PFA and ADVpp, respectively, compared with the WT polymerase.

The WT drug susceptibility of the K513N mutant polymerase observed in the activated DNA-based assay indicates that the mutation does not modify the ability of the enzyme to elongate DNA in the presence of inhibitor. However, viral DNA replication occurring during the HCMV life cycle is a complex process directly involving at least six viral proteins (Pari *et al.,* 1993a). The assay using enzymes isolated from infected cells may not fully reflect this level of complexity; therefore, we used the transient transfection/infection assay to compare drug susceptibilities of DNA synthesis of the WT and K513N recombinant virus during their normal replication cycle. In this assay, intracellular replication of a plasmid pSP50 containing the origin of HCMV lytic-phase DNA replication (*ori*Lyt) is driven by viral proteins provided in *trans* by HCMV infection (Anders *et al.,* 1992), and its sensitivity to antiviral agents can be quantitatively determined. Figure 5 shows that the plasmid replication after infection with the K513N mutant was 10- and 4-fold less susceptible to CDV and GCV, respectively, compared with plasmid replication in the presence of WT virus. In contrast, there was no difference in the susceptibility to PFA. These data suggest that the K513N mutation diminishes the sensitivity of viral DNA replication to CDV and GCV but not to PFA, which correlates with the drug susceptibility phenotype of the K513N mutant virus.

DISCUSSION

To date, selection of CDV-resistant strains during CDV therapy has not been reported; therefore, the primary goal of this work was to determine whether CDV could select for a resistant HCMV strain *in vitro*. Because it has been previously shown that HCMV strains containing UL54 mutations selected by GCV exhibit cross-resistance to CDV (Lurain *et al.,* 1992; Snoeck *et al.,* 1996; Sullivan *et al.,* 1993), we concurrently carried out a parallel selection in the presence of GCV. Genotypic analysis of the CDV- and GCV-selected strains revealed an identical K513N amino acid substitution in viral DNA polymerase. The GCV-selected virus also expressed the V812L polymerase alteration. Furthermore, it carried the M460V mutation in the UL97 gene, which has been previously detected by others in a GCV-resistant clinical isolate and was proved by marker transfer to confer a 5–6-fold decrease in susceptibility to GCV (Chou *et al.,* 1995). It has been shown in cell culture as well as *in vivo*

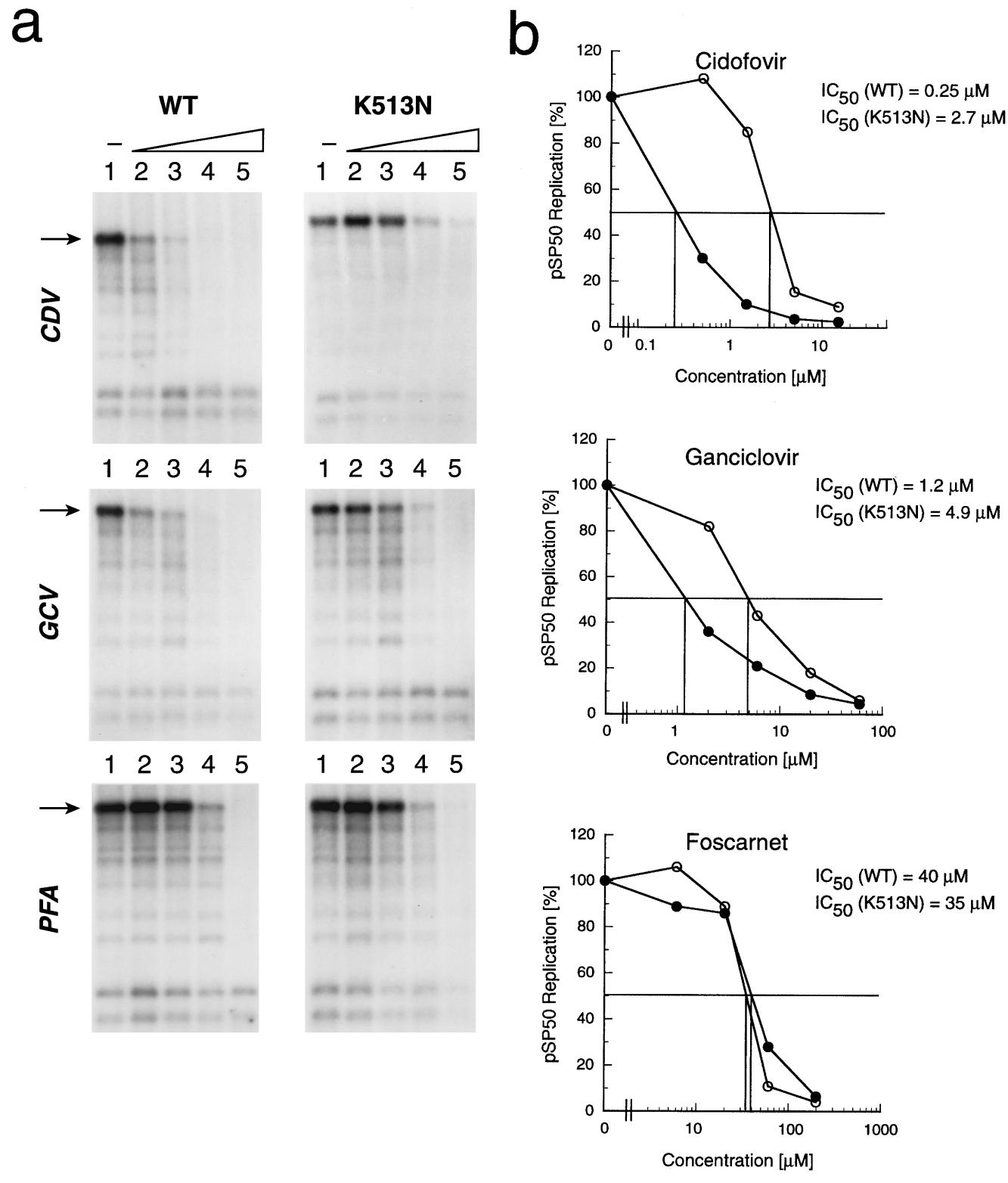

FIG. 5. Susceptibility to CDV, GCV, and PFA of pSP50 (*ori*Lyt) plasmid replication after infection with either WT or K513N mutant recombinant virus. (a) The level of pSP50 plasmid replication in the presence of various concentrations of CDV (lane 1, no drug; lanes 2–5, 0.5, 1.5, 5, and 15 μM), GCV (lane 1, no drug; lanes 2–5, 2, 6, 20, and 60 μM) and PFA (lane 1, no drug; lanes 2–5, 6, 20, 60, and 200 μM) was determined by the transient transfection/infection assay. The arrow indicates the intracellularly replicated pSP50 plasmid resistant to *Dpn*I digestion. (b) Drug dose-response curves and IC_{50} values for inhibition of pSP50 replication after infection with the WT (●) and K513N (○) recombinant viruses.

that GCV is capable of selecting for viruses with mutations in both the UL97 and UL54 gene (Sullivan *et al.,* 1993; Chou *et al.,* 1997; Smith *et al.,* 1997). However, this is the first reported example of a selection in the presence of GCV that resulted in a combination of two alterations in DNA polymerase in addition to a UL97 mutation.

Recently, a detailed study of drug resistance-associated mutations in the UL54 gene revealed that most of the alterations conferring the same drug resistance phenotype cluster within specific DNA polymerase conserved regions. Alterations located in region IV and δ-region C were found to specifically confer CDV/GCV resistance and ADV hypersensitivity (Cihlar *et al.,* 1998). Characterization of the K513N mutation located in δ-region C supports these findings. Previously, other variants of codon 513 (K513E and K513R) were identified in drug-resistant clinical isolates (Smith *et al.,* 1997). It is noteworthy that the recombinant virus expressing the K513E substitution exhibited CDV and GCV resistance and ADV hypersensitivity at similar levels as those observed for the K513N mutant recombinant virus (Cihlar *et al.,* 1998).

The V812L substitution conferring a moderate decrease in susceptibility to CDV, GCV, PFA, and ADV resides in the UL54 conserved region III (Fig. 1), in which at least four other HCMV drug resistance-associated mutations have been identified to date. Contrary to substitutions in region IV, region II, and δ-region C, the mutations located in region III confer various phenotypes ranging from decreased susceptibility to specific drugs in some cases to drug hypersensitivity in others (Cihlar *et al.,* 1998).

Unexpectedly, further investigation of the molecular mechanisms of HCMV drug resistance associated with the K513N substitution revealed no significant differences between the K513N and WT DNA polymerase with regard to their sensitivity to CDVpp and GCVppp. On the contrary, an enzyme with the V715M mutation exhibited decreased sensitivity to PFA and ADVpp but not to CDVpp and GCVppp, which is in complete agreement with the drug-susceptibility profile of the V715M mutant recombinant virus (Baldanti *et al.,* 1996; Cihlar *et al.,* 1998). Thus, drug resistance of the V715M mutant virus is likely due to decreased affinity of the mutant polymerase to the corresponding inhibitors. Although the K513N mutation did not decrease polymerase affinity for CDVpp and GCVppp, it did reduce the susceptibility to CDV and GCV of the replication of the plasmid containing HCMV *ori*Lyt sequence. Thus, it is possible that the molecular mechanisms of resistance due to the K513N mutation involves more complex interactions between the inhibitors and HCMV DNA polymerase. Previously, primer extension assays using native isolated WT polymerase revealed that CDV can be internally incorporated into a DNA primer (Xiong *et al.,* 1997b), suggesting that it may also be incorporated into viral DNA during HCMV replication. This DNA may then serve as a template in the subsequent replication cycles of the virus. However, it has been shown that DNA synthesis by the WT enzyme proceeds very inefficiently on a template with internally incorporated CDV molecules (Xiong *et al.,* 1997b). The K513N mutation may modify the ability of DNA polymerase to use such a defective template. This type of difference between the WT and K513N enzyme would not be detectable by the steady-state enzyme kinetic experiments described here.

In the second part of the work, we attempted to characterize additional functional consequences of the K513N substitution. The replication kinetic experiments disclosed an impairment in the replication capacity of the K513N mutant recombinant virus. Because the drug resistance-associated mutations at codon 513 are clinically relevant (Smith *et al.,* 1997), it would be interesting to determine the *in vivo* replication capacity of the K513N mutant virus. Unfortunately, it is not possible to address this question due to inefficient replication of HCMV laboratory strains in animal models. Notably, however, the replications of drug-resistant HSV-2 and murine cytomegalovirus selected *in vitro* by CDV both showed severely impaired pathogenicity when tested in animal models (Mendel, D. B., Tai, C. Y., Barkhimer, D. B., Kern, E. R., Chen, M. S., unpublished data; Smee *et al.,* 1995), suggesting that the K513N mutant may also replicate more slowly *in vivo*.

The K513N mutant enzyme, both *in vitro* expressed and isolated from virus-infected cells, was virtually deficient in the 3′-5′ exonuclease function. These data demonstrate that HCMV with impaired 3′-5′ exonuclease activity is still able to replicate in cell culture, suggesting that fully active 3′-5′ exonuclease may not be essential for *in vitro* virus replication. Based on the homology with other α-like DNA polymerases, it has been proposed that four amino acid segments (designated domain ExoI, ExoI′, ExoII, and ExoIII, respectively) located in the N-terminal portion of HCMV DNA polymerase form the 3′-5′ exonuclease active site (Fig. 1) (Bernad *et al.,* 1989; Blanco *et al.,* 1991; Simon *et al.,* 1991). Specific mutations within the Exo motifs of bacterial, yeast, or viral DNA polymerases can impair the 3′-5′ exonuclease function (Hall *et al.,* 1995; Kühn and Knopf, 1996; Morrison *et al.,* 1991; Simon *et al.,* 1991). However, residue 513 in HCMV polymerase is the first identified amino acid located outside the known exonuclease domains that is important for retaining the 3′-5′ exonuclease activity. The fact that this amino acid is conserved among several DNA polymerases containing δ-region C indicates that it may play a direct role in enzyme catalytic functions.

Notably, HSV-1 strains carrying specific mutations in δ-region C within the ExoIII domain of the DNA polymerase gene exhibited not only a lower replication capacity in cell culture but also significantly higher mutational frequency due to a severely impaired 3′-5′ exonuclease function (Hwang *et al.,* 1997). In addition,

site-directed mutagenesis studies of the exonuclease domains in bacterial and yeast DNA polymerases demonstrated the importance of 3′-5′ exonuclease function for the fidelity of DNA replication (Morrison *et al.,* 1991; Simon *et al.,* 1991). Based on these findings, it is believed that the 3′-5′ exonuclease activity of all replicative DNA polymerases exerts a proofreading function during the process of DNA synthesis (Echols and Goodman, 1991). Thus, the 3′-5′ exonuclease deficiency that may exist in some of the drug-resistant HCMV mutants may increase their mutational frequency and facilitate the subsequent selection of additional drug resistance-associated mutations.

In summary, this work represents a comprehensive characterization of a drug-resistant HCMV variant selected in the presence of CDV. The UL54 mutation identified in this variant markedly compromised the *in vitro* replication capacity of the mutant virus. Although the effect of drug resistance-associated UL54 mutations on the *in vivo* virus replication is not fully understood at this time, the results of this study suggest that certain HCMV UL54 mutant variants may have *in vivo* limited replication capacity. These variants may not arise readily during the antiviral therapy, and if so, they may exhibit reduced pathogenicity.

MATERIALS AND METHODS

Cells and viruses

NHDFs obtained from Clonetics (San Diego, California) were maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were not used for $>$10 passages. HCMV strain AD169, obtained from American Type Culture Collection (ATCC VR-538; Rockville, Maryland), as well as all of the generated HCMV strains, were grown in NHDFs.

Generation of recombinant mutant viruses

The recombinants were constructed by an approach based on intracellular homologous recombination of nine overlapping viral DNA fragments after their cotransfection into permissive cells (Cihlar *et al.,* 1998). Cosmids containing seven of these viral DNA fragments were kindly provided by George Kemble from Aviron (Mountain View, California). Plasmids with each of the remaining two DNA fragments (7.4 and 12 kbp) were constructed as described previously (Cihlar *et al.,* 1998). The mutations of interest were introduced into the UL54 gene residing within the 7.4-kbp DNA fragment by a two-step polymerase chain reaction (PCR) amplification procedure (Cihlar *et al.,* 1998). For introduction of the K513N mutation, a pair of mutagenic primers 5′-CGGCAACGCAATGATGACCTGTCTTAC-3′/5′-GTAAGACAGGTCATCATTGCGTTGCCG-3′ was used. The V812L mutation was incorporated into the UL54 gene using primers 5′-GGCGCTCAAATTAACGTGCAACGCTTTC-3′ and 5′-GAAAGCGTTGCACGTTAATTTGAGCGCC-3′. The underlined bases indicate the mutation sites. For generation of the K513N/V812L recombinant virus, the two mutations were introduced sequentially. After cloning the PCR products into the 7.4-kbp DNA fragment, the correct nucleotide sequence of the entire region amplified by PCR was verified by sequence analysis of both DNA strands. To generate each recombinant virus, the 7.4-kbp DNA fragment carrying the corresponding mutation was transfected by calcium phosphate precipitation into embryonic lung fibroblasts together with eight other overlapping HCMV DNA fragments. The genotype of each recombinant virus was verified by sequencing after PCR amplification of the UL54 gene.

Drug susceptibility assays

The standard plaque reduction assay in a 24-well plate format was used to determine virus sensitivities to CDV (Gilead Sciences, Foster City, CA), GCV (Hoffmann-La Roche, Nutley, New Jersey), PFA (Sigma Chemical, St. Louis, Missouri), ADV (Gilead Sciences), and LBV (Bristol-Myers Squibb, Wallingford, CT) (Cherrington *et al.,* 1996). Briefly, NHDFs infected with 30–60 plaque-forming units (pfu)/well were incubated for 5–8 days in either the absence or presence of drug at various concentrations. The plates were stained with 0.1% crystal violet in 20% methanol, and the number of plaques was visually determined and expressed as a percentage of the plaques detected in the absence of drug. The IC_{50}values were determined from the semilogarithmic plot of plaque percentage versus drug concentration.

In vitro replication kinetic experiments

The replication capacity of the WT and K513N mutant recombinants was compared based on the yield of extracellular virus after infection of NHDFs. To accurately standardize the virus input, two parallel 12-well plates were infected with 2-fold serially diluted inoculum of each virus. Beginning on day 5 after infection, aliquots of growth media were collected every 24 h from one plate infected with each virus and stored at $-70°C$. In addition, at 5–7 days after infection, parallel plates were stained with crystal violet and used for pfu determination. Media collected from wells infected with identical input of virus (50–60 pfu/well) were simultaneously titered for the yield of recombinant viruses in 24-well plates using 10-fold serial dilutions. Two independently generated WT and K513N mutant recombinants were tested in duplicate. Before the growth kinetic experiments, the correct genome structure of the recombinant viruses was verified by comparison of *Eco*RI and *Hin*dIII restriction digest profiles of purified viral DNA.

Enzymes

The WT, K513N, and V715M mutant polymerases were isolated from virus-infected NHDFs using a previously described procedure (Mar *et al.,* 1985; Xiong *et al.,* 1997b). Fifteen roller-bottles (850 cm^2) of confluent cells were infected with the individual recombinant viruses at an m.o.i. of 0.5. When most of the cells showed cytopathic effect (usually 5–8 days after infection), cells were harvested, washed in phosphate-buffered saline (PBS), and solubilized in the presence of 0.03% Nonidet-P40. After centrifugation at 100,000 *g* for 90 min, each enzyme was isolated from the supernatant by four sequential chromatographic steps on DEAE-Sephacel, phosphocellulose, heparin-agarose, and single-stranded DNA agarose. To improve the stability of the enzymes during the purification, 0.2 mg/ml bovine serum albumin (BSA) was added to each column fraction. The fractions were assayed by the filter-based assay in the presence or absence of 90 mM $(NH_4)_2SO_4$ using activated calf thymus DNA (Xiong *et al.,* 1997b). Active fractions stimulated by the presence of salt were pooled for further purification. After a final chromatography step, the enzymes were concentrated using a Centriprep-10 (Amicon, Beverly, Massachusetts) and stored in 10 mM KPO_4, pH 8.0, 1 mM EDTA, 2 mM dithiothreitol, 1 mg/ml BSA, and 30% glycerol at −70°C.

The quality of enzyme preparations was examined by immunoblot analysis. Enzymes were separated by electrophoresis on an 8% polyacrylamide gel in the presence of SDS and electroblotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, Massachusetts), which was subsequently blocked in PBS/5% dry milk (PBS-M) for 1 h, washed three times in PBS/0.05% Tween 20 (PBS-T), and incubated overnight in PBS-M with rabbit antiserum raised against HCMV DNA polymerase (kindly provided by Eng-Shang Huang, University of North Carolina at Chapel Hill). After the wash in PBS-T, the membrane was incubated in PBS-M with goat anti-rabbit antibody conjugated to alkaline phosphatase (Zymed, South San Francisco, California). After an additional wash and incubation with a chemiluminescent substrate (Amersham, Arlington Heights, Illinois), the immunoblot was exposed to a x-ray film. Quantification of the enzymes isolated from infected cells was achieved by using identical conditions, except a Cy5-conjugated donkey anti-rabbit antibody (Jackson Immunoresearch, West Grove, Pennsylvania) was used instead of the alkaline phosphatase conjugate. The relative amount of each protein was determined after scanning the immunoblot using the Storm 860 PhosphorImager system (Molecular Dynamics, Sunnyvale, California).

In addition to isolation from infected cells, the WT and K513N mutant polymerases also were expressed using the coupled *in vitro* transcription/translation system (Cihlar *et al.,* 1997). Circular plasmids pUL54–6 (Cihlar *et al.,* 1997) containing either the WT or mutant UL54 gene were simultaneously subjected to expression in the TNT coupled reticulocyte lysate system (Promega, Madison, Wisconsin) according to the manufacturer's instructions. To verify the expression of enzymes and determine their relative quantity, the transcription/translation reactions were carried out in the presence of [^{35}S]methionine (Amersham; specific activity, 1200 Ci/mmol), and their products were separated by SDS–PAGE. The fixed and dried gel was scanned using the PhosphorImager system, and the bands corresponding to the full-length UL54 proteins were quantified.

Enzymatic assays

The DNA-dependent DNA polymerase activity of the WT and K513N mutant enzyme was determined using the filter-based assay (Cihlar *et al.,* 1997). Reactions were performed in a 60-μl volume and contained polymerase buffer [20 mM Tris–HCl, pH 8.0, 90 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mg/ml BSA, 5% glycerol]; 6 μg of activated calf thymus DNA, 0.5 μM [^{3}H]dGTP (Amersham; specific activity, 37 Ci/mmol); 10 μM concentration each of dATP, dCTP, and dTTP; and the enzyme. The amount of the WT and K513N mutant polymerase in the reaction was standardized according to their relative quantifications described above. After 10-, 20-, and 30-min incubations at 37°C, aliquots of each reaction were removed and processed as described (Cihlar *et al.,* 1997). A linear time course of each reaction was generated, and relative reaction rates were determined. One unit of enzymatic activity (1 U) was defined as the amount of enzyme-catalyzing incorporation of 1 pmol of dGTP into activated calf thymus DNA per hour under the specified conditions.

Identical assay conditions were used for determination of kinetic constants. K_m values were measured in the presence of 10 μM dNTPs and various concentrations of the appropriate [^{3}H]dNTP (Amersham; specific activity, 30 Ci/mmol). Determination of K_i values for CDVpp, GCVppp, and PFA was carried out at four different inhibitor concentrations. Kinetic constants were calculated from double-reciprocal plots of initial reaction rates using KinetAsyst software (Think Technologies, San Diego, CA) based on published algorithms (Cleland, 1979).

The 3′-5′ single-stranded exonuclease activity was assayed by measuring the release of [^{3}H]dTMP from [^{3}H]oligo(dT) attached to the 3′-end of linearized plasmid DNA (Cihlar *et al.,* 1997). Reactions were performed with 0.5 μCi of substrate (5×10^5 cpm/μg of DNA) in 60 μl of polymerase buffer. After 10-, 20-, and 30-min incubations at 37°C with a standardized amount of the WT or K513N mutant enzyme, 15-μl sample aliquots were removed and processed as described (Cihlar *et al.,* 1997). Relative reaction rates were determined from a linear time course of each reaction.

Transient transfection/infection assay

The assay was carried out as described previously (Cihlar *et al.,* 1998). Briefly, the plasmid pSP50 containing the HCMV origin of lytic replication (kindly provided by Dr. Greg Pari, Hybridon, Cambridge, Massachusetts) was transfected into NHDFs by $CaPO_4$ precipitation (Pari *et al.,* 1993b). After transfection, the cells were infected with either the WT or K513N mutant recombinant virus at an m.o.i. of 10 and incubated in either the absence or presence of various concentrations of CDV, GCV, or PFA. Total DNA was purified from each sample 120 h after infection, digested with *Eco*RI and *Dpn*I, and separated on a 0.7% agarose gel. DNA was then transferred onto a Hybond-N^+ membrane (Amersham) and probed with ^{32}P-labeled pGEM cloning vector (Promega) as described previously (Cihlar *et al.,* 1998). The membrane was first exposed to x-ray film and then scanned using the PhosphorImager system. The bands corresponding to intracellularly replicated full-length pSP50 were quantified and expressed as a percentage of plasmid replication in the absence of drug. The 50% inhibitory concentration was determined for each drug from the semilogarithmic plot of the percentage of plasmid replication versus drug concentration.

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